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Allelic configuration and polysomic inheritance of highly variable microsatellites in tetraploid gynodioecious *Thymus praecox* agg.

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Abstract Polyploidy plays a pivotal role in plant evolution. However, polyploids with polysomic inheritance have hitherto been severely underrepresented in plant population genetic studies, mainly due to a lack of appropriate molecular genetic markers. Here we report the establishment and experimental validation of six fully informative microsatellite markers in tetraploid gynodioecious *Thymus praecox* agg. Sequence data of 150 microsatellite alleles and their flanking regions revealed high variation, which may be characteristic for polyploids with a reticulate evolutionary history. Understanding the patterns of mutation (indels and substitutions) in microsatellite flanking-sequences was a prerequisite for the development of co-dominant markers for fragment analyses. Allelic segregation patterns among progeny arrays from ten test crosses revealed tetrasomic inheritance in *T. praecox* agg. No evidence of frequent double reduction was detected.

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Polymerase chain reaction (PCR) based dosage effects allowed for precise assignment of allelic configuration at all six microsatellite loci. The quantification of allele copy numbers in PCR was verified by comparisons of observed and expected gametic allele frequencies and heterozygosities in test crosses. Our study illustrates how PCR based markers can provide reliable estimates of heterozygosity and, thus, powerful tools for breeding system and population genetic analyses in polyploid organisms.

Introduction

Polyploidy plays a significant role in plant evolution; a prominent example being the production of agricultural crops. Much scientific effort has been made to investigate the formation, establishment, genome organization and molecular evolution of polyploids (Bennett 2004; Soltis et al. 2004). For instance, higher selfing ability leading to better colonizing ability of polyploids relative to their diploid progenitors has been viewed to contribute to the success of polyploids (Soltis and Soltis 2000). Furthermore, population genetic theory predicts increased levels of allelic diversity and heterozygosity in polyploids (Bever and Felber 1992), which may provide a genetic buffer against inbreeding depression (Ronfort 1999; Soltis and Soltis 2000) and genetic drift (Barrett and Kohn 1991; Ronfort et al. 1998). However, empirical evidence from natural populations is still scarce and additional studies are needed to evaluate the above population genetic expectations (Ronfort 1999; Soltis and Soltis 2000; Landergott et al. 2001; Galloway et al. 2003; Lopez-Pujol et al. 2004). Empirical studies on polyploids require knowledge on their mode of inheritance (Bever

and Felber 1992; Ronfort et al. 1998; Ronfort 1999). Species of presumably allopolyploid origin show disomic inheritance, where segregation is similar to that of nonhomologous pairs of chromosomes in diploids. In contrast, autopolyploids form multivalents during meiosis resulting in polysomic inheritance with a pattern of segregation varying from random assortment of homologous chromosomes to random assortment of sister chromatids. The latter phenomenon, referred to as double reduction, occurs when there is recombination between the centromere and a given locus, which increases the production of homozygous gametes (Bever and Felber 1992).

Recently, attention has been drawn on potential effects of polyploidization on the evolution of plant sexual systems via changes in self-compatibility, sex determination or inbreeding depression (Pannell et al. 2004). For example, polyploidization may trigger the evolution of dioecy in plants via the loss of self-incompatibility (Miller and Venable 2000). Moreover, as the nuclear genome becomes multiplied while the organellar genomes do not, polyploidization also affects nuclear-cytoplasmic interactions (Wendel 2000; Pannell et al. 2004). Both cytoplasmic male-sterility factors and multiple nuclear restorers of male function are involved in the sex expression in many gynodioecious plant species where female and hermaphroditic individuals coexist in the same population (Charlesworth and Laporte 1998).

We are currently studying the evolution of nuclear-cytoplasmic gynodioecy in the tetraploid plant *Thymus praecox* agg. Selfing rates and inbreeding depression are important key parameters in the evolution of gynodioecious species with self-compatible hermaphrodites and obligatory outcrossed females (Jacobs and Wade 2003). Marker based estimates of selfing rates and heterozygosity are useful to infer inbreeding depression in natural populations of long-lived plants (Ritland 1990). Therefore, highly variable and co-dominant molecular genetic markers provide an important tool for investigations on the maintenance of nuclear-cytoplasmic gynodioecy in natural populations of *T. praecox* agg.

The use and interpretation of allozyme markers in polyploid species is often difficult (Hardy et al. 2001; Lopez-Pujol et al. 2004), and allelic diversity at allozyme loci is limited (Olson 1997; Galloway et al. 2003). In contrast, co-dominant microsatellite markers with high allelic diversity provide a powerful molecular genetic tool for studies on mating patterns and breeding systems. Unfortunately, in polyploid species, analysis of PCR based microsatellite markers is not as straightforward as in diploids. The quantification of the

copy number per allele has often been reported as impossible in species with polysomic inheritance (Esselink et al. 2004; Nybom et al. 2004; De Silva et al. 2005; Luo et al. 2006). Recent work on roses has though highlighted the potential use of PCR based dosage effects for estimating the allelic configuration at microsatellite loci in polyploid plants via MAC-PR (microsatellite DNA allele counting-peak ratios; Esselink et al. 2004; Nybom et al. 2004). Because of varying amplification intensity among different alleles in roses, Esselink et al. (2004) and Nybom et al. (2004) analyzed all alleles in pairwise comparisons by calculating the ratios between the amplification intensities of two co-occurring alleles. MAC-PR is thus a laborious approach, and fully heterozygous individuals are desirable to determine the 1:1 ratios that are used as a base line for calculations of allele quantification in partial homozygotes. However, direct co-dominant interpretation of microsatellite loci based on relative polymerase chain reaction (PCR) product intensities has also been reported in tetraploid sweet potato (Buteler et al. 1999), in lake sturgeon (McQuown et al. 2002), in alfalfa (Julier et al. 2003; Flajoulot et al. 2005) and in birch (Truong et al. 2005). The relatively novel interpretation of PCR based allele dosage effects should yet be done with caution in order to rule out potential artifacts (Wagner et al. 1994; Esselink et al. 2004). Both PCR selection caused by differential primer affinity and PCR drift resulting from random events during early cycles of PCR have been claimed to cause skewness in simultaneously amplified products (Wagner et al. 1994). To verify estimates of allele copy numbers, known parent–offspring relationships should be most helpful (Buteler et al. 1999; Nybom et al. 2004). Only allelic segregation patterns in conformance with expectations under a given mode of polyploid inheritance can verify allelic configuration estimates.

In the present study, we report the development, optimization and experimental validation of six fully informative and highly variable microsatellite markers in tetraploid gynodioecious *T. praecox* agg. Tetrasomic inheritance of the microsatellite markers was evident from controlled crosses. Precise tetraploid allelic configurations were directly determined from PCR based dosage effects at all six loci. Mendelian segregation of the microsatellite alleles confirmed the quantification of allele copy numbers in PCR. Heterozygosity was unambiguously scored in individuals from geographically remote natural populations of *T. praecox* agg. From a general viewpoint, our work outlines access to fully informative PCR based molecular markers representing a powerful tool for breeding system and population genetic analyses in polyploid species.

Materials and methods

Study species

Hybridization is very common in the genus *Thymus* (Lamiaceae), especially among tetraploid taxa (Jalas and Kaleva 1970; Stahl-Biskup and Sáez 2002). In the phylogenetically young section *Serpyllum*, the central European tetraploid representatives are morphologically highly diverse and taxonomically difficult. Jalas and Kaleva (1970) hypothesized that each of these tetraploid taxa probably originated from multiple allopolyploidization events between several diploid species. Taxonomists have classified the *Thymus* taxa of the European Alps in a wealth of subspecies, varieties and formae. In a more sensible approach, Jalas (1970) suggested to treat several tetraploid taxa of the European mountains as members of the single polymorphic species aggregate *Thymus praecox* Opiz ampl. Jalas, with five subspecies (Tutin et al. 1972). Our study populations are best assigned to *T. praecox* Opiz ssp. *polytrichus* (A. Kerner ex Borbás) Jalas. However, as the subspecific boundaries are vague (Jalas 1970, 1971), we will only refer to *T. praecox* agg. in the following. Individuals of gynodioecious *T. praecox* agg. are long-lived and form large cushions. Hermaphrodites are self-compatible. The species is widespread in the European Alps from subalpine to alpine altitudes and is widely found on rocky surfaces and in pastures.

The individuals of *T. praecox* agg. used in the present work originated from study populations in the Swiss Alps from the geographically remote regions Flimsenstein [F], Goms [G], Langwies [L], Piora valley [P], Säntis [S] and Zwinglipass [Z], from both low [L] and high [H] altitudinal sites each. Precise locations are available from the authors upon request. Individuals were labeled by sex [F=female; H=hermaphrodite] and identified by a number per sexual phenotype. Individual PLF05 thus refers to the study female number five from a low altitudinal population in the Piora valley. For permanently marked females in natural populations, seeds from open pollination were collected and grown in a greenhouse. From a controlled crossing experiment of individuals transplanted to two experimental fields, full-sib families were available to investigate the mode of inheritance in *T. praecox* agg. (see below for sample sizes).

Additionally, two individuals from one population of each of four closely related *Thymus* taxa were collected: the tetraploid *T. praecox* Opiz ssp. *arcticus* (E. Durand) Jalas from Ireland, the diploids *T. pulegioides* L. from the Swiss Alps and *T. serpyllum* L. from Hungary (all section *Serpyllum*) and the Mediterra-

nean diploid *T. vulgaris* L. (section *Thymus*) from France.

Development of microsatellite markers

We isolated microsatellite loci in *T. praecox* agg. from a partial genomic library, screened on nylon membranes following the procedure of Estoup and Martin (1996). From two individuals of *T. praecox* agg., genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen), further purified with the Prep-A-Gene Purification System (Biorad) and pooled for digestion with *AluI*, *HaeIII* and *RsaI* (Boehringer Mannheim). Size-selected fragments (500–1,000 bp) were ligated in pUC18, and ultra-competent cells (Stratagene) were transformed. A total of 2,500 recombinant clones were transferred to LB-agar plates and consecutively lifted onto nylon membranes (Boehringer Mannheim). Hybridization was carried out with digoxigenin-labeled probes [(AC)₁₂, (AG)₁₂, (AAT)₈ and (TCT)₈], prepared with the DIG oligonucleotide tailing kit (Roche Molecular). A total of 19 hybridizing clones were detected with the DIG Luminescent Detection Kit (Roche Molecular) and used as PCR templates for sequencing with the universal pUC/M13 primers.

Different microsatellite repeat motifs were found in 12 sequences (0.5% of the recombinant clones; GeneBank accession nos. AM087131–AM087142) and primers were designed for sites in the flanking regions suitable for PCR amplification. Amplifications were carried out in 10 µl reaction volumes (see below for optimized reaction conditions). The PCR products were separated on Spreadex gels (EL 300-600, Elchrom Scientific), stained with ethidium bromide and recorded under UV light. Initial amplification products of microsatellite loci in *T. praecox* agg. pointed to three main problems that needed to be addressed for further optimization of the markers: (1) The performance in PCR varied markedly among individuals suggesting differences in the quality of template DNA; (2) the magnitude of size differences of alleles suggested the presence of size variation in the microsatellite flanking regions; (3) there was evidence of null alleles due to mutations within primer sites.

A series of DNA extractions testing different protocols and additional precipitation and purification reagents was carried out in order to improve DNA quality (thyme is well known for its various secondary metabolites and intraspecific chemical polymorphism; Stahl-Biskup and Sáez 2002). Best results were obtained from the DNeasy Plant Mini Kit (Qiagen) with the following modifications to the manufacturer's protocol: a maximum of 10 mg of silica dried leaf

material was used. In the protein precipitation step, 20 µl of 25% PVP (polyvinylpyrrolidone) was added in order to remove phenolic compounds. After the binding of DNA to the minicolumn, it was washed twice with 600 µl of a desalting wash buffer (400 mM NaCl, 20 mM Tris (pH 7.5), 2 mM EDTA (pH 7.5) and 50% EtOH (v/v); Prep-A-Gene Purification System, Biorad) and once with 500 µl of 80% EtOH. Extracted DNA was stored in Tris-buffer.

In order to isolate alleles of microsatellites together with their adjacent flanking regions for sequencing, gel pieces of well-separated bands were picked from Spreadex gels under UV light using 1,000 µl pipette tips. Gel pieces were ejected into 10 µl TE, incubated over night and 1 µl of the resulting solution was used as template for direct re-amplification of isolated alleles in 15 µl reaction volumes. Re-amplification products were purified with the Prep-A-Gene Purification System (Biorad) and sequenced in both directions using BigDyeTM Terminator Ready Reaction Kit 2.0 on an ABI 3100 automated sequencer (Applied Biosystems). Sequencing signals tended to decrease or, in some cases, broke down after the microsatellite repeat array. Sequences were aligned manually in SEQUENCHER (version 3.1.1; Gene Codes Corporation). New, mostly degenerate primers were designed for conservative sites close enough to the microsatellite locus in order to exclude insertions/deletions in the flanking regions. Since ghost bands on nondenaturing Spreadex gels did not allow an unambiguous interpretation of tetraploid allelic patterns, HPLC purified labeled primers (6FAM and HEX, Microsynth; NED, Applied Biosystems) were used to score the most promising loci on an ABI 3100 automated sequencer (36 cm capillaries, POP-6, Rox 400 HD internal size standard, GENESCAN version 3.7; Applied Biosystems). Electropherogram peak heights were used to estimate the copy number per allele. PCR conditions (annealing temperature, annealing and extension times, as well as concentrations of primers, MgCl₂ and BSA) were optimized for best detection of allelic configurations. As several locus-individual combinations still failed to resolve tetraploid allelic configuration (null alleles), additional alleles were isolated using more remote primers in combination with degenerate ones located close to the microsatellite. Alleles were sequenced from the remote primer only. After sequence alignment, new, even higher degenerate primers were designed (Table 1; Electronic Supplementary Material I).

Optimized PCR amplifications were carried out using 1 ng of template DNA in 10 µl reaction volumes of 0.15 mM of each dNTP, 5% DMSO, 1× polymerase buffer, 0.025 U/µl *Taq* DNA polymerase (Sigma) and

locus-specific concentrations of primers, MgCl₂ and BSA (Table 1). Cycles were run on Genius and TC-412 thermal cyclers (Techne) with initial denaturation for 3 min at 96°C, locus-specific (Table 1) number of cycles of 35 s at 95°C, locus-specific annealing time and temperature, locus-specific elongation time at 72°C and final extension of 20 min at 60°C. The cooling ramp was set to 0.5°C/s, starting from 65°C for loci D257 and D347 and from 60°C for the remaining four loci. Loci D346 and D347 had the most degenerate primers (Table 1) with considerably different optimal annealing temperatures predicted per oligonucleotide variant (Microsynth). Therefore, these two loci were also tested for improved amplification performance with the Multiplex PCR Kit (Qiagen). This PCR Master mix contains a synthetic factor MP that stabilizes specifically bound primers and enables efficient extension of all primers in a reaction. We added DMSO and BSA as above to 10 µl reaction volumes. Initial denaturation of 15 min at 95°C was applied to activate the HotStarTaq DNA polymerase. Clean amplification products at decreased annealing temperature and improved estimates of allelic dosage were obtained for locus D347 for which the Multiplex PCR Kit was chosen as the standard protocol (Table 1).

Inheritance in controlled crosses, confirmation of allelic configuration estimates and resolution power of the microsatellite markers

To investigate segregation patterns at the six microsatellite loci, we used seedlings of ten test crosses (Table 2): Two hermaphrodites from two different populations were (1) selfed (crosses A and F), (2) outcrossed with a hermaphrodite from within the same population (B and G) and (3) used as a pollen donor in a within population cross with a female (C and H). The two females were additionally crossed (4) with hermaphrodites from a different population within study region P (D and I) and (5) from the geographically remote region Z (E and K). Our crossing design provided four large half-sib families, those of the parental individuals PHH06, PHF14, PLH19 and PLF05, which were used in three different crosses each (Table 2). A total of 75 progenies were genotyped. One seedling of cross A proved to be outcrossed with an unknown father and was therefore excluded, and one seedling of cross B was a self and thus included in cross A for analyses.

Maternal and paternal gamete genotypes were determined for each progeny and each locus from the allelic composition of parental genotypes (Electronic Supplementary Material II). The mode of inheritance (disomic vs. polysomic) was inferred from the pairing pattern of parental alleles among gametes. Under

Table 1 Characterisation, amplification conditions and allele diversity of six microsatellite markers in *Thymus praecox* agg.

Locus	Primer Label	Primer sequence (5')	Primer (μM)	Annealing Temperature (°C)	Time (s)	Elongation time (s)	MgCl ₂ (mM)	BSA (μg)	No. of Repeat cycles	Fragment size (bp) ^a	No. of alleles ^a	H ₀ ^a	Detected outcrosses ^b
C405	F1	GGTGAAGATGGGATTGTGCTAC	0.2	50.0	65	14	2.0	5.0	25	CT	148–184	19	0.92
	R4	GASGATAAGACTTGMGTAMAASC	0.8										17
D257	F3b	TCTCCRAASMCCTAACTATTC	0.6	57.5	85	06	1.5	5.0	26	AG	73–142	24	0.90
	R2	CGATCCATGGCTTGACATGTGC	0.2										19
D346	F4	CATMAWCCCCATAAWAGCARCAC	1.2	46.0	95	07	1.5	5.0	25	AG	82–146	24	0.92
	R2d	CTCTCAAMACACAYACCAASC	0.9										18
D347	F3b	YACACACACAGYGDAGGTG	1.1	51.0	85	14	3.0	5.0	26	(GA)(GAA)	104–163	39	0.96
	R2b	GTGCCTCCYCTATWCATCAC	0.7										20
E070	F4	CCCATGTTGYAGTTACTGCG	0.4	46.0	70	10	2.0	7.5	26	GA	125–155	17	0.83
	R6	GCCATTTTCATYYMTMTCTCC	0.9										16
E089	F3	AARAGAAAGAGRAGAAGAA	0.7	46.0	65	08	2.0	5.0	24	GAA	124–142	6	0.54
	R4	GAAGAAGTAAACCTGAAATCC	0.2										9

H₀ observed heterozygosity^aBased on fragment analyses of 40 partly related individuals from natural populations (10 females with two offspring each; 10 parental individuals of controlled crosses)^bOut of 20 obligatory outcrossed offspring of 10 females from natural populations

polysomic inheritance, the formation of a homozygous gamete from a single copy parental allele would provide direct evidence for double reduction. In a larger data set including all the parental alleles, excess gamete homozygosity would provide indirect evidence for double reduction.

In order to check the accuracy of the quantification of allele copy number in PCR, we recorded the allele frequencies among all gametes per parental individual and locus (including frequencies among progenies derived from self pollination even though the genotypes of the gametic phase were not known in these families). These observed gametic allele frequencies were tested for significant departure from those expected from parental allelic configurations under random chromosome segregation by means of Chi-squared tests (Table 3). Furthermore, for each parental individual and locus, we recorded the observed frequency and calculated the expected frequencies of homozygous gametes under both random chromosome and random chromatid segregation (with a maximal frequency of double reduction of one-seventh in the latter case; Bever and Felber 1992). Wilcoxon signed-ranks tests were used to test for systematic deviation of observed from expected frequencies of homozygous gametes per locus.

To check the applicability of the microsatellite markers to geographically remote samples of *T. praecox* agg., ten females, one each of a low and high altitudinal population from regions F, L, P, S and Z were genotyped. Per female, two offspring of open pollinated origin (=obligatory outcrossed) were genotyped. This also permitted to assess the efficiency of the markers in resolving outcrossing events in natural populations. The relationship between the number of different alleles per locus and both the number of detected outcrosses and the mean observed heterozygosity (Table 1) was studied with Spearman rank correlation coefficients. Observed individual heterozygosity was scored following Bever and Felber (1992), with the genotypes weighted by 1 minus the probability of any two alleles being identical by descent ($AAAA=0$, $AAAB=0.5$, $AABB=0.667$, $AABC=0.833$ and $ABCD=1$). Statistical analyses were performed in SPSS (version 10.0.8 for Mac; SPSS Inc.).

Results

Sequence variation in flanking regions and within microsatellite repeat arrays

A total of 150 alleles out of 6 microsatellite loci of *T. praecox* agg. was sequenced. These alleles were iso-

lated from 9 to 20 individuals per locus, issued from 11 geographically remote natural populations of *T. praecox* agg. Full sequences of alleles are listed in Electronic Supplementary Material I, along with adjacent flanking-sequences. The sequence characterization of the six loci revealed high variation both in flanking regions and in microsatellite repeat arrays.

Within microsatellite flanking regions, substitutions as well as indels (insertions/deletions) were frequent at all six loci. In several loci, one flanking region was relatively conservative while the other was variable. Different flanking-sequence variants were detected within and among individuals of *T. praecox* agg., and particular variants were repeatedly found in samples from geographically remote regions (e.g. an indel mutation in locus D257 and a series of base substitutions in loci C405 and E070). As expected from the commonly high mutation rate within repeat arrays (Dettman and Taylor 2004), different flanking-sequence variants were associated with multiple repeat alleles. However, divergent flanking-sequence variants also shared identical repeat alleles (e.g. in loci C405, E070 and E089). Primer pairs could be designed to exclude flanking-sequence indels from marker fragments for three loci. However, immediately adjacent to the dinucleotide repeat microsatellites C405 and E070, short deletions of an even number of bases were detected confusing variation in repeat number in fragment analyses. In locus D347, several indels remained in the marker fragment. Base substitutions within flanking regions were so frequent that most of the final primers designed for microsatellite marker fragment amplification had to be degenerate (Table 1).

Within microsatellite arrays, mutations other than changes in the number of repeat units were common. An insertion caused odd numbers of base pairs in a

part of the alleles of the dinucleotide microsatellite E070. Indels were also found in microsatellite D347. Base substitutions within repeat arrays were detected in the microsatellites D346, D347 and, most prominently, in locus D257. Furthermore, the duplication of repeats with substitutions (imperfect repeats) was observed in locus D257. Out of a total of 29 repeat alleles sequenced from locus D257, the number of different lengths was 12, but the number of unique sequences was 23.

Allele size diversity and resolving power of the microsatellite markers

Allelic diversity based on marker fragment length was determined for each of the six microsatellite loci in 40 individuals from 10 natural populations of *T. praecox* agg. (Table 1). The trinucleotide microsatellite E089 showed the lowest variation in repeat number with a total of six different alleles. The four dinucleotide repeat loci C405, D257, D346 and E070 displayed 17–24 different alleles, and the compound microsatellite D347 showed the highest variation in repeat number with 39 different alleles in the 40 individuals studied. The number of different alleles per locus was positively correlated with its power to resolve outcrossing events in natural populations ($r_s=0.986$, $P < 0.001$; Table 1). All of the 10 females and their 20 obligatory outcrossed offspring from different populations were genetically distinguished by means of the single, highly polymorphic locus D347. The same result was obtained from the combined analysis of the four dinucleotide repeat loci. These highly variable microsatellite markers are thus valuable to investigate selfing rates of hermaphrodites in natural populations of *T. praecox* agg.

Table 2 Controlled crosses and estimates of multilocus heterozygosity within progeny arrays of *Thymus praecox* agg.

Cross	Mother × father	Treatment	Progenies ^a	MLH ₀ ^b
A	PHH06 × PHH06	Selfed	8	0.591
B	PHH06 × PHH01	Outcrossed within population	6	0.744
C	PHF14 × PHH06	Outcrossed within population	8	0.783
D	PHF14 × PLH04	Outcrossed among populations within region	8	0.717
E	PHF14 × ZHH18	Outcrossed among regions	8	0.854
F	PLH19 × PLH19	Selfed	8	0.642
G	PLH19 × PLH08	Outcrossed within population	8	0.800
H	PLF05 × PLH19	Outcrossed within population	8	0.750
I	PLF05 × PHH02	Outcrossed among populations within region	6	0.811
K	PLF05 × ZLH05	Outcrossed among regions	6	0.889

^aNumber of progenies genotyped per family

^bEstimate of multilocus heterozygosity calculated from observed heterozygosities at five microsatellite loci (locus D347 was excluded due to missing data caused by a null allele; Table 3)

Table 3 Comparison of observed and expected gametic allele frequencies and heterozygosities at six microsatellite loci in ten controlled crossing families of *Thymus praecox* agg.

Locus	Parent		Gametes inherited both via pollen and ovules								
	Individual	Genotype	Allele frequencies ^a			Ascertained genotypes ^c	Homozygous				
			Expected (RCeS)	Observed	$P(\chi^2)^b$		O	RCeS		RCdS	
								E	$P(Z)^d$	E	$P(Z)^d$
C405	PHH06	<i>ccel</i>	22c-11e-11l	19c-7e-18l	0.042	8	1	1.3		2.3	
	PHH01	<i>acde</i>				6	0	0.0		0.9	
	PHF14	<i>ackm</i>	10a-10c-10k-10m	14a-7c-11k-8m	0.392	20	0	0.0		2.9	
	PLH04	<i>aaac</i>									
	ZHH18	<i>iiij</i>	8i-8j	9i-7j		8	3	2.7		3.4	
	PLH19	<i>aade</i>	32a-16d-16e	30a-15d-19e	0.687	15	1	2.5		4.3	
	PLH08	<i>aacj</i>	8a-4c-4j	11a-2c-3j		8	3	1.3		2.3	
	PLF05	<i>cefi</i>	10c-10e-10f-10i	9c-11e-12f-8i	0.801	20	0	0.0		2.9	
	PHH02	<i>bcbj</i>	3b-3c-3g-3j	2b-4c-2g-4j		6	0	0.0		0.9	
	ZLH05	<i>abhj</i>	3a-3b-3h-3j	4a-4b-2h-2j		6	0	0.0		0.9	
					97	8	7.8	0.854	20.6	0.015	
D257	PHH06	<i>accj</i>	14a-28c-14j	15a-30c-11j	0.651	8	0	1.3		2.3	
	PHH01	<i>ccgj</i>									
	PHF14	<i>ccfi</i>	23.5c-11.75f-11.75i	25c-8f-14i	0.422	24	5	4.0		6.9	
	PLH04	<i>ackl</i>	4a-4c-4k-4l	4a-3c-4k-5l		8	0	0.0		1.1	
	ZHH18	<i>ee fi</i>	7.5e-3.75f-3.75i	8e-3f-4i		8	1	1.3		2.3	
	PLH19	<i>accf</i>	12a-24c-12f	14a-24c-10f	0.717	8	0	1.3		2.3	
	PLH08	<i>bcdh</i>	4b-4c-4d-4h	3b-5c-4d-4h		8	0	0.0		1.1	
	PLF05	<i>aacc</i>	12a-12c	10a-14c	0.414	12	4	4.0		5.1	
	PHH02	<i>adgm</i>	3a-3d-3g-3m	4a-2d-1g-5m		6	0	0.0		0.9	
	ZLH05	<i>bekn</i>	3b-3e-3k-3n	2b-4e-4k-2n		6	0	0.0		0.9	
					88	10	12.0	0.269	22.9	0.007	
D346	PHH06	<i>cdem</i>	14c-14d-14e-14m	11c-14d-18e-13m	0.603	14	0	0.0		2.0	
	PHH01	<i>bbeg</i>	6b-3e-3g	7b-2e-3g		6	1	1.0		1.7	
	PHF14	<i>acde</i>				24	0	0.0		3.4	
	PLH04	<i>aaeh</i>				8	2	1.3		2.3	
	ZHH18	<i>bdfϕ^c</i>	4b-4d-4f-4 ϕ	4b-6d-3f-3 ϕ		8	0	0.0		1.1	
	PLH19	<i>aijn</i>	13a-13i-13j-13n	7a-15i-11j-19n	0.104	16	0	0.0		2.3	
	PLH08	<i>abjk</i>				8	0	0.0		1.1	
	PLF05	<i>ajjo</i>	6a-12j-6o	5a-13j-6o	0.882	12	2	2.0		3.4	
	PHH02	<i>abfl</i>	3a-3b-3f-3l	4a-3b-3f-2l		6	0	0.0		0.9	
	ZLH05	<i>befh</i>	3b-3e-3f-3h	4b-4e-3f-1h		6	0	0.0		0.9	
					108	5	4.3	0.317	19.1	0.005	
D347	PHH06	<i>flnp</i>	15f-15l-15n-15p	15f-21l-15n-9p	0.187	14	0	0.0		2.0	
	PHH01	<i>dkpu</i>	3d-3k-3p-3u	4d-1k-5p-2u		6	0	0.0		0.9	
	PHF14	<i>joux</i>	12j-12o-12u-12x	10j-13o-11u-14x	0.841	24	0	0.0		3.4	
	PLH04	<i>dh ty</i>	4d-4h-4t-4y	2d-5h-5t-4y		8	0	0.0		1.1	
	ZHH18	<i>cemv</i>	4c-4e-4m-4v	4c-5e-4m-3v		8	0	0.0		1.1	
	PLH19	<i>gm nϕ^c</i>	13.8g-13.8m-13.8n-13.8 ϕ	13g-20m-8n-14 ϕ	0.152	16	0	0.0		2.3	
	PLH08	<i>irsw</i>	4i-4r-4s-4w	4i-4r-6s-2w		8	0	0.0		1.1	
	PLF05	<i>flps</i>	10f-10l-10p-10s	8f-8l-12p-12s	0.659	20	0	0.0		2.9	
	PHH02	<i>bmos</i>	3b-3m-3o-3s	4b-2m-3o-3s		6	0	0.0		0.9	
	ZLH05	<i>afkq</i>	3a-3f-3k-3q	4a-1f-3k-4q		6	0	0.0		0.9	
					116	0	0.0	1.000	16.6	0.005	
E070	PHH06	<i>abbc</i>	15a-30b-15c	20a-28b-12c	0.301	14	2	2.3		4.0	
	PHH01	<i>deei</i>	3d-6e-3i	4d-5e-3i		6	0	1.0		1.7	
	PHF14	<i>defi</i>	12d-12e-12f-12i	17d-9e-8f-14i	0.212	24	0	0.0		3.4	
	PLH04	<i>befh</i>	4b-4e-4f-4h	4b-5e-5f-2h		8	0	0.0		1.1	
	ZHH18	<i>eejk</i>	8e-4j-4k	8e-5k-3j		8	0	1.3		2.3	
	PLH19	<i>bdee</i>	12b-12d-24e	11b-11d-26e	0.846	8	2	1.3		2.3	
	PLH08	<i>deeh</i>									
	PLF05	<i>bbdd</i>	17b-17d	18b-16d	0.732	12	5	4.0		5.1	
	PHH02	<i>dffg</i>	3d-6f-3g	3d-5f-4g		6	0	1.0		1.7	
	ZLH05	<i>eehl</i>	6e-3h-3l	6e-2h-4l		6	0	1.0		1.7	
					92	9	12.0	0.197	23.4	0.007	

Table 3 continued

Locus	Parent		Gametes inherited both via pollen and ovules								
	Individual	Genotype	Allele frequencies ^a			Ascertained genotypes ^c	Homozygous				
			Expected (RCeS)	Observed	$P(\chi^2)^b$		O	RCeS		RCdS	
								E	$P(Z)^d$	E	$P(Z)^d$
E089	PHH06	cccc	52c	52c		14	14	14.0		14.0	
	PHH01	accc	3a-9c	3a-9c		6	3	3.0		3.4	
	PHF14	accc	12.5a-37.5c	14a-36c	0.624	24	11	12.0		13.7	
	PLH04	cccc	16c	16c		8	8	8.0		8.0	
	ZHH18	bccd	4b-8c-4d	3b-10c-3d		8	2	1.3		2.3	
	PLH19	accc	12a-36c	12a-36c	1.000	8	3	4.0		4.6	
	PLH08	cccd	12c-4d	11c-5d		8	3	4.0		4.6	
	PLF05	aacc									
	PHH02	accc									
	ZLH05	accc									
						76	44	46.3	0.131	50.6	0.042

E expected, *O* observed, *RCdS* random chromatid segregation, *RCeS* random chromosome segregation

^aIncluding allele frequencies among progenies derived from selfing in crosses A and F

^bChi-squared tests were calculated for larger data sets only, i.e. for parents that were used in multiple crosses (Table 2)

^cTotal number of gametes for which the allelic configuration was determined

^dWilcoxon signed-ranks test for deviation of observed from expected frequency of homozygous gametes

^eNull allele

Inheritance of microsatellite markers

Parental alleles were randomly combined in gamete genotypes at all six microsatellite loci as inferred from ten test crosses (e.g. in male gametes of individual ZHH18 at locus E089; Electronic Supplementary Material II). This showed that inheritance in *T. praecox* agg. is tetrasomic. No direct evidence for double reduction was detected at any of the six loci, based on observations of heterozygous gametes (C405: $n=93$; D257: $n=86$; D346: $n=108$; D347: $n=116$; E070: $n=95$; E089: $n=36$). Furthermore, at all of the six loci, heterozygosity among gametes was significantly higher than expected under maximal double reduction (i.e. under random chromatid segregation; Table 3).

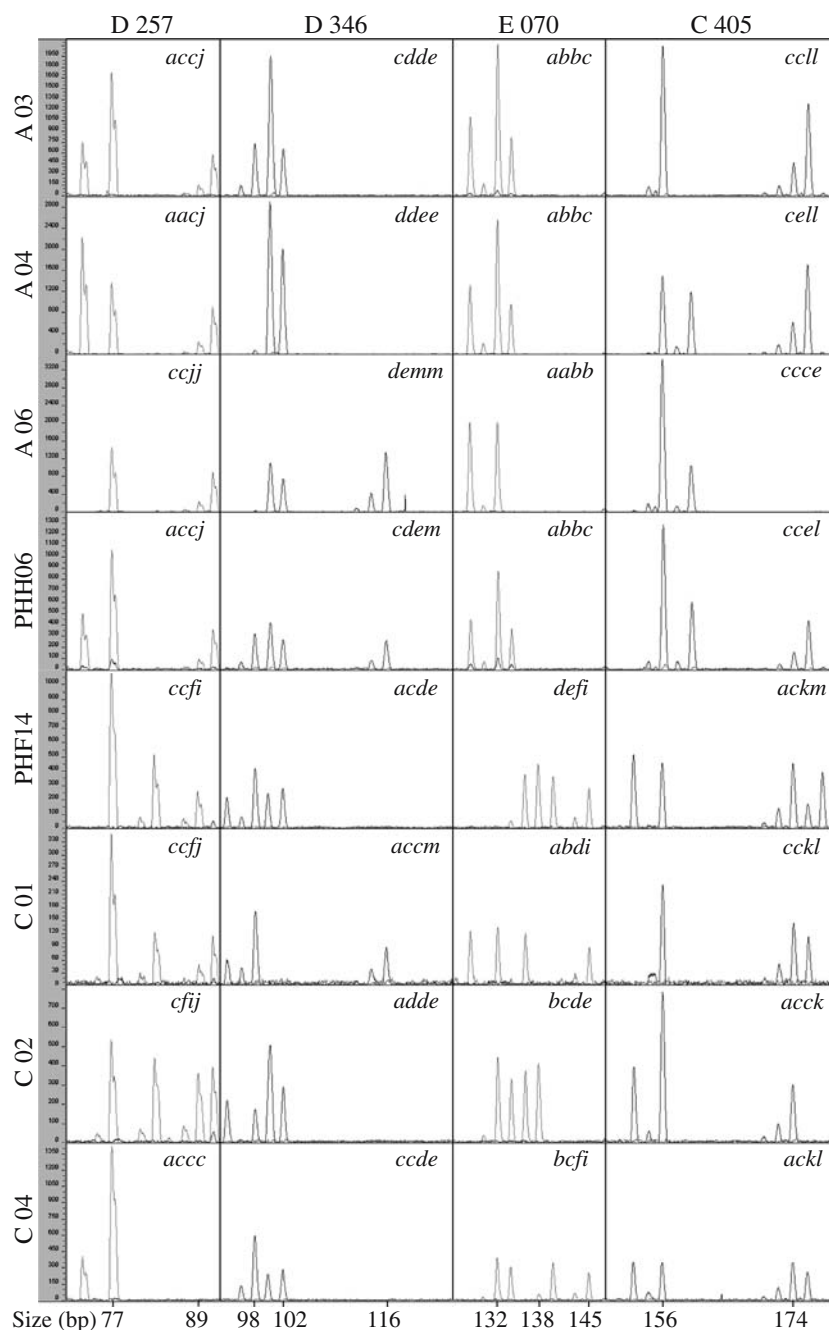
Assignment of microsatellite allelic configurations: heterozygosity estimates

Direct determination of tetraploid allelic configurations from PCR product intensities was possible for all six microsatellite loci and almost all of the 114 individuals of *T. praecox* agg. genotyped in the current study (Fig. 1). Electropherogram peak heights of the six microsatellite markers in the 84 individuals analyzed from controlled crosses are listed in Electronic Supplementary Material II. Best homogeneity among amplification product intensities was obtained by applying annealing temperatures from the lower

boundaries of those predicted for the different variants of a degenerate primer pair (except for locus D257), by adjusting the primer concentrations for the degree of degeneration and by optimization of annealing time (Table 1). Longer repeat alleles tended to display lower amplification intensities due to kinetic effects of PCR (e.g. in the progeny array of cross K at the loci D257 and E070; Electronic Supplementary Material II). Kinetic effects could be reduced by optimization of extension times and of BSA- and $MgCl_2$ -concentrations (Table 1). Increased concentrations of BSA and $MgCl_2$, however, did also increase the amount of stutter products.

Stutter fragments, one and two repeat units shorter than the actual allele, were more pronounced the higher the repeat number was (Fig. 1). Therefore, stutter peak heights were added up to allele peak heights in order to estimate allelic dosage in loci C405, D257, D346 and D347 (Electronic Supplementary Material II). Corrections for the slope of kinetic effects did further improve estimates of allelic dosage (data not shown), but such calculations were not necessary for the assignment of allelic configurations in the present data set. Some differential amplification intensity among alleles was detected at the three loci D257, D346 and D347 (Electronic Supplementary Material II). Allelic differences in amplification intensity were consistent within individuals and among their offspring. However, amplification intensity could

Fig. 1 Electropherograms of four microsatellite loci in eight individuals of tetraploid *Thymus praecox* agg. showing PCR based dosage effects of allele copy number. Progenies A03, A04 and A06 were derived from self-fertilization of hermaphrodite PHH06. Progenies C01, C02 and C04 were derived from controlled crosses of female PHF14 with pollen from PHH06. Amplification products of the four loci C405, D257, D346 and E070 were multiplexed for fragment analysis (peaks of the internal size standard are suppressed in electropherograms)



vary within allele size among individuals. For example, allele D257-*g* was underamplified in individual PHH02, but not so in PHH01 (Electronic Supplementary Material II). Similarly, allele D346-*d* was overamplified in PHH06, but not so in PHF14, and allele D346-*a* was underamplified in PLH04 and PLH19, but less so in PLF05 and PLH08. The MAC-PR approach was needed for locus D346 to assign the allelic configuration of individual D05. A weak erroneous product of 98 bp in marker D346 also co-amplified in some individuals

(Electronic Supplementary Material II) and needed to be taken into account for the assignment of allelic configuration (e.g. in individuals PHF14 and C02; Fig. 1).

The analysis of gamete genotypes as inferred from the test crosses confirmed that the quantification of allele copy numbers in parental individuals was accurate. Allele frequencies among gametes were in good congruence with those predicted from parental allelic configurations at all of the six microsatellite loci (ex-

cept for PHH06 for locus C405; Table 3). The observed heterozygosity among gametes was not significantly different from that expected under random chromosome segregation at any of the six loci (Table 3).

High levels of heterozygosity were observed among the 40 individuals from natural populations of *T. praecox* agg. (Table 1). Mean observed heterozygosity was positively correlated with allele diversity per locus ($r_s=0.868$, $P=0.025$). Full heterozygotes were frequently detected at all loci other than E089. The fully homozygous state was found only in one individual for locus D257 and in six individuals for locus E089. Only in three individuals, evidence of null alleles was observed (locus D346 in individual ZHH18; locus D347 in PLH19; locus E070 in an offspring of SLF08). Controlled crosses revealed an average reduction in observed heterozygosity of 0.20 in the progenies derived from selfing of hermaphrodites PHH06 and PLH19 as compared to their maternal half-sibs derived from outcrossing (Table 2, Fig. 1). The observed loss of heterozygosity due to inbreeding was close to the expected value of 0.17 per generation (under random chromosome segregation; Bever and Felber 1992).

Cross-amplification in related thyme species

All of the six markers successfully and consistently amplified microsatellite loci in *T. praecox* ssp. *arcticus*, *T. pulegioides*, *T. serpyllum* and *T. vulgaris* (except for E089 in the latter species). Allelic configurations were in conformance with the ploidy levels of these thyme species (Tutin et al. 1972). Detected allele sizes were in the range of those found in *T. praecox* agg.

Discussion

The development of fully informative microsatellite markers in tetraploid *T. praecox* agg. had two major technical obstacles, which had their cause in evolutionary features of polyploid plant species. First, *T. praecox* agg. showed polysomic inheritance. Fully informative genetic markers should thus reliably estimate allele copy number at a given locus. Tetrasomic inheritance points to an autopolyploid origin of *T. praecox* agg. In contrast, Jalas and Kaleva (1970) supposed an allopolyploid origin of the species based on morphological variation. Independent of the type of polyploidization, however, recurrent formation and reticulate evolution seem to be the rule rather than the exception in polyploid plant species (Soltis et al. 2004). Not unexpectedly, therefore, high genetic diversity in microsatellite loci and flanking regions was a second

major characteristic complicating the establishment of microsatellite markers in *T. praecox* agg.

High variation in microsatellite flanking-sequences and within repeat arrays

Flanking regions of microsatellites have been reported to show mutation rates of an order of magnitude lower than those found within microsatellite repeat arrays (Dettman and Taylor 2004). The variation in microsatellite flanking regions may thus be useful to infer phylogenetic relationships among closely related species (Rossetto et al. 2002; Dettman and Taylor 2004). However, we detected high within-species variation in microsatellite flanking regions in *T. praecox* agg., and geographically remote populations shared divergent flanking-sequence variants. Our findings may be characteristic for a polyploid species with a reticulate evolutionary history. The successful cross-amplification of the six microsatellite markers in related thyme species indicates that *T. praecox* agg. could have accumulated different flanking-sequence variants present in the genus. The accumulation of sequence diversity may further point to multiple origins of the tetraploid aggregate species and/or to introgression from other tetraploid thyme species. Our molecular data are in agreement with reports on extraordinarily high morphological variation and a mixing of otherwise either southerly or northerly distributed biochemical compounds in *T. praecox* agg. from the European Alps (Jalas 1970; Jalas and Kaleva 1970; Bischof-Deichnik et al. 2000).

Technically, indel mutations in flanking regions should be excluded from marker fragments, because they can cause differences in fragment length that are not attributable to differences in repeat number at the microsatellite. Additionally, base substitutions within primer sites can cause amplification failure and null alleles.

Understanding the patterns of mutation within hypervariable microsatellite loci is crucial for a biologically meaningful interpretation of markers (Balloux and Lugon-Moulin 2002; Dettman and Taylor 2004). Sequence data provided evidence for multiple factors causing marker allele size homoplasy in *T. praecox* agg. (Electronic Supplementary Material I). (1) In several loci, the association of repeat alleles of identical state with different flanking-sequence variants indicated that it was rather unlikely that all copies of the same repeat allele were identical by descent. (2) Indels in the flanking-sequence next to the microsatellite repeat array could not be avoided for three loci (C405, D347 and E070). (3) Substitution mutations within repeat arrays

caused size homoplasy at three loci (D257, D346 and D347). Allele size homoplasy would seriously interfere with estimates of genetic differentiation (Balloux and Lugon-Moulin 2002) and leads to underestimation of actual heterozygosity. High allele size diversity at microsatellite loci (e.g. D257 and D347) will in turn decrease the probability for homoplasy within individuals. Indeed, the high levels of heterozygosity observed in individuals from natural populations of *T. praecox* agg. suggested that underestimation of heterozygosity should be of a minor magnitude. Both microsatellite allele size diversities and observed heterozygosities were markedly higher in tetraploid *T. praecox* agg. (Table 1) than those reported in polyploid *Rosa* sect. *Caninae* (Nybom et al. 2004) and *Betula pubescens* ssp. *tortuosa* (Truong et al. 2005).

Our findings corroborate recommendations to sequence microsatellite alleles of a representative subsample of individuals in order to assess their suitability for addressing particular questions (Buteler et al. 1999; Dettman and Taylor 2004). We directly isolated microsatellite alleles from high-resolving electrophoretic gels for subsequent sequencing. This approach was efficient to assess the patterns of mutation in microsatellite loci and their flanking-sequences for our purpose, i.e. for the design of markers that should reliably estimate relative levels of heterozygosity among individuals.

Allelic configuration and inheritance of microsatellites

The high variability of microsatellites facilitated the determination of putative gamete genotypes at the six study loci for most of the ten test crosses. The random combination of parental alleles in gametes rejected disomic inheritance and demonstrated tetrasomic inheritance in *T. praecox* agg. Random chromosome and random chromatid segregation are endpoints of a continuum in polysomic inheritance and most loci probably fall somewhere between these two extremes (Bever and Felber 1992). We did not attempt to obtain precise estimates of double reduction in the current study. Larger sample sizes would be required for this purpose as well as knowledge on selective forces possibly affecting heterozygosity in a gynodioecious species (Bailey et al. 2003; Hansson and Westerberg 2002). However, the results from controlled crosses indicated that the frequency of double reduction was, at best, low at all of the six microsatellite loci, i.e. that inheritance was close to random chromosome segregation.

Under polysomic inheritance, various allelic configurations and heterozygosity states are possible at a

given locus (Bever and Felber 1992). Thus, the fundamental prerequisite for the assignment of microsatellite allelic configurations in a polyploid species with polysomic inheritance is the accurate reproduction of allele copy number by PCR. It has been hypothesized, however, that amplification bias may occur among simultaneously amplified fragments due to PCR drift and/or PCR selection, which would impede the interpretation of PCR based dosage effects (Wagner et al. 1994). Full repeatability of relative allele peak intensities between independent PCRs ruled out the occurrence of random PCR drift in our amplifications. In contrast, differential amplification intensities among alleles provided evidence for PCR selection resulting from differential affinity of degenerate primer variants. These effects of PCR selection could largely be reduced by optimization of PCR conditions.

Most recently, MAC-PR has been proposed as an alternative approach to deal with differential amplification intensities among alleles in polyploid plant species (Esselink et al. 2004). A basic assumption of the MAC-PR method is the repeatability of relative allelic amplification intensities among individuals and, thus, homology of microsatellite marker alleles within a species. Sequence data highlighted that this assumption is likely violated in *T. praecox* agg. due to potential repeat allele size homoplasy. Indeed, for the few alleles showing skewed amplification intensity, skewness markedly varied among individuals. Therefore, MAC-PR may be useful to improve determination of allelic configuration within crossing families, but it would not be generally applicable for estimating allelic dosage in screening natural populations of *T. praecox* agg.

The validation of co-dominant molecular markers finally comprises the conformance of allelic segregation patterns with those predicted under a certain mode of inheritance, which is tetrasomic with nearly random chromosome segregation in the case of *T. praecox* agg. No major departure from Mendelian tetrasomic inheritance with random chromosome segregation was detected in allelic segregation patterns among the six microsatellite loci studied. Thus, the set of markers yields reliable estimates of heterozygosity at these microsatellite loci and is, despite the minor drawbacks mentioned above, appropriate to address evolutionary questions in *T. praecox* agg.

Conclusions

Polyploids have hitherto been largely neglected in plant population genetic studies, mainly due to a lack of suitably variable and fully informative molecular

genetic markers (Esselink et al. 2004; Soltis et al. 2004). Our present study demonstrates that PCR techniques are appropriate to determine allele copy numbers under polysomic inheritance. Knowledge on sequence variation in microsatellite loci and their flanking regions was though a prerequisite for the establishment of co-dominant microsatellite markers in tetraploid *T. praecox* agg. High levels of variation other than changes in the number of repeat units should generally be expected in microsatellites of species with a reticulate evolutionary history, i.e. in many polyploid plants.

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